

# POLYNUCLEOTIDE CONSTRUCTS FOR INCREASED LYSINE PRODUCTION

## CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims the right of priority under 35 U.S.C. §119 to U.S. Provisional Appl. No. 60/267,183 filed on February 8, 2001, the entirety of which is incorporated by reference herein.

## BACKGROUND OF THE INVENTION

### Field of the Invention

[0001] The invention relates to production of lysine, and provides several isolated polynucleotide molecules useful for the production of L-lysine. One such polynucleotide encodes an aspartate kinase (ask), an aspartate-semialdehyde dehydrogenase (asd) and a dihydrodipicolinate reductase (dapB). Other polypeptides encode ask, asd, dapB and a diaminopimelate dehydrogenase (ddh); ask, asd, dapB, ddh and an ORF2 polypeptide; and ask, asd, dapB, ddh, ORF2 and a diaminopimelate decarboxylase (lysA). The invention further provides methods of making and using the polynucleotides, and methods to increase the production of L-lysine.

### Related Art

[0002] L-lysine is an important economic product obtained principally by industrial-scale fermentation utilizing the Gram positive *Corynebacterium glutamicum*, *Brevibacterium flavum* and *Brevibacterium lactofermentum* (Kleemann, A., *et. al.*, Amino Acids, in ULLMANN'S ENCYCLOPEDIA OF

INDUSTRIAL CHEMISTRY, vol. A2, pp.57-97, Weinham: VCH-Verlagsgesellschaft (1985)).

**[0003]** The stereospecificity of the amino acids produced by fermentation makes the process advantageous compared with synthetic processes; generally L-form amino acids are produced by the microbial fermentation process. The production of L-lysine and other amino acids through fermentation, utilizing cheap carbon sources such as molasses, glucose, acetic acid and ethanol, is a relatively inexpensive means of production.

**[0004]** Several fermentation processes utilizing various strains isolated for auxotrophic or resistance properties are known in the art for the production of L-lysine: U.S. Patent No. 2,979,439 discloses mutants requiring amino acid supplementation (homoserine, or L-methionine and L-threonine); U.S. Patent No. 3,700,557 discloses mutants having a nutritional requirement for L-threonine, L-methionine, L-arginine, L-histidine, L-leucine, L-isoleucine, L-phenylalanine, L-cystine, or L-cysteine; U.S. Patent No. 3,707,441 discloses a mutant having a resistance to an L-lysine analog; U.S. Patent No. 3,687,810 discloses a mutant having both an ability to produce L-lysine and a resistance to bacitracin, penicillin G or polymyxin; U.S. Patent No. 3,708,395 discloses mutants having a nutritional requirement for homoserine, L-threonine, L-threonine and L-methionine, L-leucine, L-isoleucine or mixtures thereof and a resistance to L-lysine, L-threonine, L-isoleucine or analogs thereof; U.S. Patent No. 3,825,472 discloses a mutant having a resistance to an L-lysine analog; U.S. Patent No. 4,169,763 discloses mutant strains of *Corynebacterium* that produce L-lysine and are resistant to at least one of aspartic analogs and sulfa drugs; U.S. Patent No. 5,846,790 discloses a mutant strain able to produce L-glutamic acid and L-lysine in the absence of any biotin action-suppressing agent; and U.S. Patent No. 5,650,304 discloses a strain belonging to the genus *Corynebacterium* or *Brevibacterium* for the production of L-lysine that is resistant to 4-N-(D-alanyl)-2,4-diamino-2,4-dideoxy-L-arabinose 2,4-dideoxy-L-arabinose or a derivative thereof.

[0005] A considerable amount is known regarding the biochemical pathway for L-lysine synthesis in *Corynebacterium* species (recently reviewed by Sahm *et al.*, *Ann. N. Y. Acad. Sci.* 782: 25-39 (1996)). Entry into the L-lysine pathway begins with L-aspartate (see Figure 1), which itself is produced by transamination of oxaloacetate. A special feature of *C. glutamicum* is its ability to convert the L-lysine intermediate piperidine 2,6-dicarboxylate to diaminopimelate by two different routes, i.e. by reactions involving succinylated intermediates or by the single reaction of diaminopimelate dehydrogenase. Overall, carbon flux into the pathway is regulated at two points: first, through feedback inhibition of aspartate kinase by the levels of both L-threonine and L-lysine; and second through the control of the level of dihydrodipicolinate synthase. Therefore, increased production of L-lysine can be obtained in *Corynebacterium* species by deregulating and increasing the activity of these two enzymes.

[0006] More recent developments in the area of L-lysine fermentative production involve the use of molecular biology techniques to augment L-lysine production. The following examples are provided: U. S. Patent Nos. 4,560,654 and 5,236,831 disclose an L-lysine producing mutant strain obtained by transforming a host *Corynebacterium* or *Brevibacterium* species microorganism which is sensitive to S-(2-aminoethyl)-cysteine with a recombinant DNA molecule wherein a DNA fragment conferring both resistance to S-(2-aminoethyl)-cysteine and L-lysine producing ability is inserted into a vector DNA; U. S. Patent No. 5,766,925 discloses a mutant strain produced by integrating a gene coding for aspartokinase, originating from coryneform bacteria, with desensitized feedback inhibition by L-lysine and L-threonine, into chromosomal DNA of a *Corynebacterium* species bacterium harboring leaky type homoserine dehydrogenase or a *Corynebacterium* species deficient in homoserine dehydrogenase gene; increased L-lysine production is obtained by gene amplification by way of a plasmid vector or utilizing a gene replacement strategy. European Patent Applications EP 0 811 682 A2 and EP 0 854 189 A2 both provide for increased production of L-lysine in *Corynebacterium* species by way of gene amplification based on plasmid copy number.

## SUMMARY OF THE INVENTION

[0007] It is an object of the invention to provide an isolated polynucleotide molecule, referred herein as the KDB polynucleotide, comprising a nucleic acid molecule encoding an aspartate kinase (ask) polypeptide; a nucleic acid molecule encoding an aspartate-semialdehyde dehydrogenase (asd) polypeptide and a nucleic acid molecule encoding a dihydrodipicolinate reductase (dapB) polypeptide. The polynucleotide may further comprise a nucleic acid encoding a complete or truncated diaminopimelate dehydrogenase (ddh) polypeptide (the KDBH polynucleotide), or a nucleic acid encoding a complete or truncated ORF2 polypeptide (the KDB2 polynucleotide). In addition, the invention provides an isolated polynucleotide molecule, referred herein as the KDB2HL polynucleotide, comprising a nucleic acid molecule encoding an ask, asd, dapB, ddh, ORF2 and diaminopimelate decarboxylase (lysA) polypeptides, in which the ddh, ORF2 and lysA polypeptides may be complete or truncated. In a preferred embodiment, a polynucleotide molecule of the invention further comprises a P1 promoter adjacent to the 5' end of the nucleotide molecule encoding lysA.

[0008] It is further the object of the invention to provide a method of increased L-lysine production in a host cell by transforming a host cell with the polynucleotide molecules described above. According to the method of the present invention, the isolated polynucleotide molecules described above are stably integrated into a chromosome of the host cell, or are maintained as extrachromosomal DNA, such as a plasmid, and the transformed host cells are selected for increased L-lysine production.

## BRIEF DESCRIPTION OF THE FIGURES

- [0009] Figure 1. A schematic of the L-lysine biosynthetic pathway in *Corynebacterium glutamicum*.
- [0010] Figure 2 A, B. The nucleotide (SEQ ID NO:1) and amino acid sequence (SEQ ID NO:2) of *ask* (ATCC 21529 sequence).
- [0011] Figure 3 A, B. The nucleotide (SEQ ID NO:3) and amino acid sequence (SEQ ID NO:4) of *asd* (ATCC 21529 sequence).
- [0012] Figure 4. The nucleotide (SEQ ID NO:5) and amino acid sequence (SEQ ID NO:6) of *dapB* (NRRL-B11474).
- [0013] Figure 5 A, B. The nucleotide (SEQ ID NO:7) and amino acid sequence (SEQ ID NO:8) of *ddh* (NRRL-B11474).
- [0014] Figure 6. The nucleotide (SEQ ID NO: 9) and amino acid sequence (SEQ ID NO: 10) of ORF2.
- [0015] Figure 7 A, B, C. The nucleotide (SEQ ID NO: 11) and amino acid sequence (SEQ ID NO: 12) of *lysA*.
- [0016] Figure 8. The nucleotide (SEQ ID NO: 13) and amino acid sequence (SEQ ID NO: 14) of truncated ORF2.
- [0017] Figure 9. The nucleotide sequence (SEQ ID NO: 15) of the P1 promoter, the first promoter of the *argS-lysA* operon from pRS6.
- [0018] Figure 10. Comparison of the aspartokinase (*ask*) amino acid sequence from ATCC13032, N13 and ATCC21529.
- [0019] Figure 11A and B. A schematic of the construction of the pDElia2<sub>FCS</sub>-KDB construct.
- [0020] Figure 12. A schematic of the construction of the pK184-KDBH construct.
- [0021] Figure 13. A schematic of the construction of the pDElia2<sub>FCS</sub>-KDB2 construct.
- [0022] Figure 14 A, B. A schematic of the construction of the pDElia2<sub>FCS</sub>-KDB2HP1L construct.

## DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

### A. Definitions

[0023] In order to provide a clear and consistent understanding of the specification and claims, including the scope to be given such terms, the following definitions are provided. It is also to be noted that the term "a" or "an" entity, refers to one or more of that entity; for example, "a polynucleotide," is understood to represent one or more polynucleotides.

[0024] **Auxotroph.** As used herein, the term refers to a strain of microorganism requiring for growth an external source of a specific metabolite that cannot be synthesized because of an acquired genetic defect.

[0025] **Amino Acid Supplement.** As used herein, the term refers to an amino acid required for growth and added to minimal media to support auxotroph growth.

[0026] **Chromosomal Integration.** As used herein, the term refers to the insertion of an exogenous DNA fragment into the chromosome of a host organism; more particularly, the term is used to refer to homologous recombination between an exogenous DNA fragment and the appropriate region of the host cell chromosome.

[0027] **High Yield Derivative.** As used herein, the term refers to strain of microorganism that produces a higher yield from dextrose of a specific amino acid when compared with the parental strain from which it is derived.

[0028] **Host Cell.** As used herein, the term "host cell" is intended to be interchangeable with the term "microorganism." Where a difference is intended, the difference will be made clear.

[0029] **Isolated Nucleic Acid Molecule.** As used herein, the term is intended to mean a nucleic acid molecule, DNA or RNA, which has been removed from its native environment. For example, recombinant DNA molecules contained in a vector are considered isolated for the purposes of the present invention. Further

examples of isolated DNA molecules include recombinant DNA molecules maintained in heterologous host cells or purified (partially or substantially) DNA molecules in solution. Isolated RNA molecules include *in vivo* or *in vitro* RNA transcripts of the DNA molecules of the present invention. Isolated nucleic acid molecules according to the present invention further include such molecules produced synthetically.

[0030] **Lysine Biosynthetic Pathway Genes.** As used herein, the term "lysine biosynthetic pathway gene(s)" is meant to include those genes and genes fragments encoding peptides, polypeptides, proteins, and enzymes, which are directly involved in the synthesis of lysine. These genes can be identical to those which naturally occur within a host cell and are involved in the synthesis of lysine within that host cell. Alternatively, there can be modifications or mutations of such genes, for example, the genes can contain modifications or mutations which do not significantly affect the biological activity of the encoded protein. For example, the natural gene can be modified by mutagenesis or by introducing or substituting one or more nucleotides or by removing nonessential regions of the gene. Such modifications are readily performed by standard techniques.

[0031] **Lysine Biosynthetic Pathway Protein.** As used herein, the term "lysine biosynthetic pathway protein" is meant to include those peptides, polypeptides, proteins, and enzymes, which are directly involved in the synthesis of lysine from aspartate. Also included are amino acid sequences as encoded by open reading frames (ORF), where the ORF is associated with a lysine biosynthetic pathway operon. These proteins can be identical to those which naturally occur within a host cell and are involved in the synthesis of lysine within that host cell. Alternatively, there can be modifications or mutations of such proteins, for example, the proteins can contain modifications or mutations which do not significantly affect the biological activity of the protein. For example, the natural protein can be modified by mutagenesis or by introducing or substituting one or more amino acids, preferably by conservative amino acid substitution, or by removing nonessential regions of the protein. Such modifications are readily

performed by standard techniques. Alternatively, lysine biosynthetic proteins can be heterologous to the particular host cell. Such proteins can be from any organism having genes encoding proteins having the same, or similar, biosynthetic roles.

[0032] **Mutagenesis.** As used herein, the term refers to a process whereby a mutation is generated in DNA. With "random" mutagenesis, the exact site of mutation is not predictable, occurring anywhere in the genome of the microorganism, and the mutation is brought about as a result of physical damage caused by agents such as radiation or chemical treatment. rDNA mutagenesis is directed to a cloned DNA of interest, and it can be random or site-directed.

[0033] **Mutation.** As used herein, the term refers to a one or more base pair change, insertion or deletion, or a combination thereof, in the nucleotide sequence of interest.

[0034] **Operably Linked.** As used herein, the term "operably linked" refers to a linkage of polynucleotide elements in a functional relationship. A nucleic acid is "operably linked" when it is placed into a functional relationship with another nucleic acid sequence. For instance, a promoter or enhancer is operably linked to a coding sequence if it affects the transcription of the coding sequence. Operably linked means that the DNA sequences being linked are typically contiguous and, where necessary, join two protein coding regions, contiguous and in reading frame. However, since enhancers generally function when separated from the promoter by several kilobases and intronic sequences can be of variable lengths, some polynucleotide elements can be operably linked but not contiguous.

[0035] **Operon.** As used herein, the term refers to a contiguous portion of a transcriptional complex in which two or more open reading frames encoding polypeptides are transcribed as a multi-cistronic messenger RNA, controlled by a cis-acting promoter and other cis-acting sequences necessary for efficient transcription, as well as additional cis acting sequences important for efficient transcription and translation (*e.g.*, mRNA stability controlling regions and transcription termination regions). The term generally also refers to a unit of gene



expression and regulation, including the structural genes and regulatory elements in DNA.

[0036] **Parental Strain.** As used herein, the term refers to a strain of host cell subjected to some form of treatment to yield the host cell of the invention.

[0037] **Percent Yield From Dextrose.** As used herein, the term refers to the yield of amino acid from dextrose defined by the formula  $[(\text{g amino acid produced} / \text{g dextrose consumed}) * 100] = \% \text{ Yield}$ .

[0038] **Phenotype.** As used herein, the term refers to observable physical characteristics dependent upon the genetic constitution of a host cell.

[0039] **Promoter.** As used herein, the term "promoter" has its art-recognized meaning, denoting a portion of a gene containing DNA sequences that provide for the binding of RNA polymerase and initiation of transcription and thus refers to a DNA sequence capable of controlling the expression of a coding sequence or functional RNA. Promoter sequences are commonly, but not always, found in the 5' non-coding regions of genes. In general, a coding sequence is located 3' to a promoter sequence. Sequence elements within promoters that function in the initiation of transcription are often characterized by consensus nucleotide sequences. The promoter sequence consists of proximal and more distal upstream elements (enhancers). As used herein, the term "endogenous promoter" refers to a promoter sequence which is a naturally occurring promoter sequence in that host microorganism. The term "heterologous promoter" refers to a promoter sequence which is a non-naturally occurring promoter sequence in that host microorganism. The heterologous occurring promoter sequence can be from any prokaryotic or eukaryotic organism. A synthetic promoter is a nucleotide sequence, having promoter activity, and not found naturally occurring in nature.

[0040] Promoters can be derived in their entirety from a native gene, or be hybrid promoters. Hybrid promoters are composed of different elements derived from different promoters found in nature, or even comprise synthetic DNA segments. Hybrid promoters can be constitutive, inducible or environmentally responsive.

[0041] Useful promoters include constitutive and inducible promoters. Many such promoter sequences are known in the art. See, for example, U.S. Pat. Nos. 4,980,285; 5,631,150; 5,707,828; 5,759,828; 5,888,783; 5,919,670, and, *Sambrook, et al., Molecular Cloning: A Laboratory Manual*, 2nd Ed., Cold Spring Harbor Press (1989). Other useful promoters include promoters which are neither constitutive nor responsive to a specific (or known) inducer molecule. Such promoters can include those that respond to developmental cues (such as growth phase of the culture), or environmental cues (such as pH, osmoticum, heat, or cell density, for example).

[0042] Examples of environmental conditions that can affect transcription by inducible promoters include anaerobic conditions, elevated temperature, or the presence of light. It is understood by those skilled in the art that different promoters can direct the expression of a gene in different cell types, or in response to different environmental conditions. Promoters which cause a gene to be expressed in most cell types at most times are commonly referred to as "constitutive promoters." It is further recognized that since in most cases the exact boundaries of regulatory sequences have not been completely defined, DNA fragments of different lengths can have identical or similar promoter activity.

[0043] **Relative Growth.** As used herein, the term refers to a measurement providing an assessment of growth by directly comparing growth of a parental strain with that of a progeny strain over a defined time period and with a defined medium.

B. Microbiological and Recombinant DNA Methodologies

[0044] The present invention relates to a KDB polynucleotide, which comprises a nucleic acid encoding an ask polypeptide, a nucleic acid molecule encoding an asd polypeptide and a nucleic acid molecule encoding a dapB polypeptide, wherein "K" represents a nucleotide sequence encoding the ask polypeptide; "D"

represents a nucleotide sequence encoding the asd polypeptide; and “B” represents a nucleotide sequence encoding the dapB polypeptide.

**[0045]** In one embodiment the present invention relates to an isolated KDB polynucleotide molecule comprising:

1. a nucleic acid molecule encoding an aspartate kinase (ask) polypeptide;
2. a nucleic acid molecule encoding an aspartate-semialdehyde dehydrogenase (asd) polypeptide; and
3. a nucleic acid molecule encoding a dihydrodipicolinate reductase (dapB) polypeptide.

**[0046]** In another embodiment, the KDB polynucleotide molecule consists essentially of a nucleic acid molecule encoding an ask polypeptide, a nucleic acid molecule encoding an asd polypeptide and a nucleic acid molecule encoding a dapB polypeptide.

**[0047]** The present invention also relates to a KDBH polynucleotide, which comprises a nucleic acid encoding an ask polypeptide, a nucleic acid molecule encoding an asd polypeptide, a nucleic acid molecule encoding a dapB polypeptide and a nucleic acid molecule encoding a ddh polypeptide, wherein “K” represents a nucleotide sequence encoding the ask polypeptide; “D” represents a nucleotide sequence encoding the asd polypeptide; “B” represents a nucleotide sequence encoding the dapB polypeptide; and “H” represents a nucleotide sequence encoding the ddh polypeptide.

**[0048]** In one embodiment, the KDBH polynucleotide molecule additionally comprises a nucleic acid encoding a complete or truncated ORF2 polypeptide.

**[0049]** The present invention also relates to a KDB2 polynucleotide, which comprises a nucleic acid encoding an ask polypeptide, a nucleic acid molecule encoding an asd polypeptide, a nucleic acid molecule encoding a dapB polypeptide and a nucleic acid molecule encoding an ORF2 polypeptide, and wherein “K” represents a nucleotide sequence encoding the ask polypeptide; “D” represents a nucleotide sequence encoding the asd polypeptide; “B” represents a nucleotide

sequence encoding the dapB polypeptide; and "2" represents a nucleotide sequence encoding the ORF2 polypeptide.

[0050] The present invention also relates to a KDB2HL polynucleotide, which comprises a nucleotide encoding an ask polypeptide, a nucleic acid molecule encoding an asd polypeptide, a nucleic acid molecule encoding a dapB polypeptide, a nucleic acid molecule encoding an ORF2 polypeptide, a nucleic acid molecule encoding a ddh polypeptide and a nucleic acid molecule encoding a lysA polypeptide. In a preferred embodiment, the KDB2HL polynucleotide molecule also comprises a P1 promoter adjacent to the 5' end of the nucleic acid encoding the lysA polypeptide.

[0051] In a preferred embodiment, the polynucleotide molecules of the present invention do not comprise any nucleic acid molecules encoding any lysine pathway polypeptides other than ask, asd, dapB, ddh, ORF2 and lysA.

[0052] In one embodiment, an ask polypeptide is defined as a polypeptide having the enzymatic activity of bacterial aspartate kinase. Bacterial aspartate kinase enzymatic activity converts L-aspartate to L-aspartylphosphate. In a preferred embodiment, an ask polypeptide would have the enzymatic activity of aspartate kinase from ATCC21529. In a preferred embodiment, the isolated ask amino sequence disclosed in SEQ ID NO:2 possesses unique properties with respect to feedback resistance of ask enzyme activity to accumulated levels of L-lysine and L-threonine in the culture medium. When compared to the DNA sequences of other *Corynebacterium glutamicum ask-asd* gene sequences, a threonine to isoleucine change at amino acid residue 380 which results in resistance to feedback inhibition is observed. Other amino acid changes at residue 380 can also result in decreased ask enzyme sensitivity to L-threonine and/or L-lysine.

[0053] An asd polypeptide is defined as a polypeptide having the enzymatic activity of aspartate-semialdehyde dehydrogenase. Aspartate-semialdehyde dehydrogenase enzymatic activity converts L-aspartylphosphate to L-aspartatesemialdehyde. In a preferred embodiment, an asd polypeptide would

have the enzymatic activity of aspartate-semialdehyde dehydrogenase from ATCC21529.

[0054] A *dapB* polypeptide is defined as a polypeptide having the enzymatic activity of dihydrodipicolinate reductase. Dihydrodipicolinate reductase enzymatic activity converts L-2,3-dihydrodipicolinate to L-piperidine-2,6-dicarboxylate. In a preferred embodiment, a *dapB* polypeptide would have the enzymatic activity of dihydrodipicolinate reductase from NRRL-B11474.

[0055] A *ddh* polypeptide is defined as a polypeptide having the enzymatic activity of diaminopimelate dehydrogenase. Diaminopimelate dehydrogenase enzymatic activity converts L-piperidine-2,6-dicarboxylate to D,L-diaminopimelate. In a preferred embodiment, a *ddh* polypeptide would have the enzymatic activity of diaminopimelate dehydrogenase from NRRL-B11474.

[0056] A *lysA* polypeptide is defined as a polypeptide having the enzymatic activity of diaminopimelate decarboxylase. Diaminopimelate decarboxylase activity converts D,L-diaminopimelate to L-lysine. In a preferred embodiment, a *lysA* polypeptide would have the enzymatic activity of diaminopimelate decarboxylase from ASO19.

[0057] *Ask*, *asd*, *dapB*, *ddh*, *ORF2* and *lysA* polypeptides encoded by the polynucleotide molecules of the present invention can be truncated forms of the polypeptides encoded by the genomic copies of the ATCC21529 *ask* and *asd* genes, the NRRL-B11474 *dapB*, *ddh*, *ORF2* genes and the ASO19 *lysA* gene.

[0058] It should be noted that in addition to the indicated polypeptide sequences encoded by the isolated nucleic acid sequences represented by "K", "D", "B," "H," "2" and "L," these isolated nucleic acid sequences can also include native promoter elements for the operons represented therein. Thus, the *ask-asd* sequences can include the respective native *ask-asd* operon elements, and the *dapB* and *ddh* sequences can include their respective native promoter elements. The preferred promoter for the nucleotide molecule encoding the *lysA* polypeptide is the P1 promoter, the first promoter from the *argS-lysA* operon.

[0059] The invention as provided herein utilizes some methods and techniques that are known to those skilled in the arts of microbiology and recombinant DNA technologies. Methods and techniques for the growth of bacterial cells, the introduction of isolated DNA molecules into host cells, and the isolation, cloning and sequencing of isolated nucleic acid molecules, etc., are a few examples of such methods and techniques. These methods and techniques are described in many standard laboratory manuals, such as Davis *et al.*, *Basic Methods In Molecular Biology* (1986), J.H. Miller, *Experiments in Molecular Genetics*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York (1972); J.H. Miller, *A Short Course in Bacterial Genetics*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York (1992); M. Singer and P. Berg, *Genes & Genomes*, University Science Books, Mill Valley, California (1991); J. Sambrook, E.F. Fritsch and T. Maniatis, *Molecular Cloning: A Laboratory Manual*, 2d ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York (1989); P.B. Kaufman *et al.*, *Handbook of Molecular and Cellular Methods in Biology and Medicine*, CRC Press, Boca Raton, Florida (1995); *Methods in Plant Molecular Biology and Biotechnology*, B.R. Glick and J.E. Thompson, eds., CRC Press, Boca Raton, Florida (1993); and P.F. Smith-Keary, *Molecular Genetics of Escherichia coli*, The Guilford Press, New York, NY (1989), all of which are incorporated herein by reference in their entireties.

[0060] In a preferred embodiment, a nucleic acid molecule encoding an ask polypeptide would be at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% identical to SEQ ID NO:1. A nucleic acid molecule encoding an asd polypeptide would be at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% identical to SEQ ID NO:3. a nucleic acid molecule encoding a dapB polypeptide would be at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% identical to SEQ ID NO:5, a nucleic acid molecule encoding a ddh polypeptide would be at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% identical to SEQ ID NO:7, a nucleic acid encoding an ORF2 polypeptide would be at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or

100% identical to SEQ ID NO:9 and a nucleic acid encoding a lysA polypeptide would be at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% identical to SEQ ID NO:11 or the complement thereof. In a preferred embodiment the nucleic acid sequence of a P1 promoter would be at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% identical to SEQ ID NO:15. In one embodiment, a nucleic acid encoding a truncated ORF2 polypeptide would be at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% identical to SEQ ID NO:13.

**[0061]** As one skilled in the art would know, any strain of *Corynebacterium* species, particularly that of *Corynebacterium glutamicum*, can be utilized for the isolation of nucleic acid molecules that will be used to amplify the number of chromosomally located amino acid biosynthetic pathway genes. Particularly preferred strains include: NRRL-B11474, ATCC 21799, ATCC 21529, ATCC 21543, and E12.

**[0062]** As a practical matter, whether any particular nucleic acid sequence is at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% identical to, for instance, a nucleotide sequence consisting of SEQ ID NO:1, SEQ ID NO:3; SEQ ID NO:5; SEQ ID NO:7; SEQ ID NO:9; SEQ ID NO:11; SEQ ID NO:13; SEQ ID NO:15, or a complementary sequence thereof, can be determined conventionally using sequence analysis computer programs such as a OMIGA® Version 2.0 for Windows, available from Oxford Molecular, Ltd. (Oxford, U.K.). OMIGA uses the CLUSTAL W alignment algorithm using the slow full dynamic programming alignment method with default parameters of an open gap penalty of 10 and an extend gap penalty of 5.0, to find the best alignment between two nucleotide sequences. When using CLUSTAL W or any other sequence alignment program to determine whether a particular sequence is, for instance, 95% identical to a reference sequence according to the present invention, the parameters are set, of course, such that the percentage of identity is calculated over the full length of the reference nucleotide sequence such that gaps, mismatches, or insertions of up to 5% of the total number of nucleotides in the reference sequence are allowed.

Other sequence analysis programs, known in the art, can be used in the practice of the invention.

[0063] Unless otherwise indicated, all nucleotide sequences described herein were determined using an automated DNA sequencer (such as the Model 373 from Applied Biosystems, Inc.), and all amino acid sequences of polypeptides encoded by DNA molecules described herein were predicted by translation of the relative DNA sequence. Therefore, as is known in the art, for any DNA sequence determined by this automated approach, any nucleotide sequence determined herein can contain some errors. Nucleotide sequences determined by automation are typically at least about 90% identical, more typically at least about 95% to at least about 99.9% identical to the actual nucleotide sequence of the sequenced DNA molecule. The actual sequence can be more precisely determined by other approaches including manual DNA sequencing methods well known in the art.

[0064] It is known in the art that amino acids are encoded at the nucleic acid level by one or more codons (code degeneracy). It is also known in the art that choice of codons may influence expression of a particular amino acid sequence (protein, polypeptide, etc.). Thus, the invention is further directed to nucleic acid molecules encoding the ask amino acid sequence of SEQ ID NO:2 wherein the nucleic acid molecule comprises any codon known to encode a particular amino acid. Likewise, the invention is directed to KDB, KDBH, KDB2 and KDB2HL polynucleotides comprising nucleic acid sequences which comprise alternative codons in order to optimize expression of the protein or polypeptide.

[0065] It will be recognized in the art that some amino acid sequences of the invention can be varied without significant effect of the structure or function of the proteins disclosed herein. Variants included can constitute deletions, insertions, inversions, repeats, and type substitutions so long as enzyme activity is not significantly affected. Guidance concerning which amino acid changes are likely to be phenotypically silent can be found in Bowie, J.U., *et al.*, "Deciphering the Message in Protein Sequences: Tolerance to Amino Acid Substitutions," *Science* 247:1306-1310 (1990).



[0066] It is preferred that the polypeptides obtained by the expression of the polynucleotide molecules of the present invention would have at least approximately 80%, 90%, 95%, 96%, 97%, 98%, 99% or 100% identity to one or more amino acid sequences selected from the group comprising SEQ ID No: 2, 4, 6, 8, 10, 12 and 14. A truncated ORF2 polypeptide has at least about 25% of the full length of an ORF2 polypeptide, preferably the ORF2 polypeptide of SEQ ID NO: 10. In one embodiment, a truncated ORF2 polypeptide has the sequence of SEQ ID NO: 14. By a polypeptide having an amino acid sequence at least, for example, 95% "identical" to a reference amino acid sequence of a polypeptide is intended that the amino acid sequence of the claimed polypeptide is identical to the reference sequence except that the claimed polypeptide sequence can include up to five amino acid alterations per each 100 amino acids of the reference amino acid of the polypeptide. In other words, to obtain a polypeptide having an amino acid sequence at least 95% identical to a reference amino acid sequence, up to 5% of the amino acid residues in the reference sequence can be deleted or substituted with another amino acid, or a number of amino acids up to 5% of the total amino acid residues in the reference sequence can be inserted into the reference sequence. These alterations of the reference sequence can occur at the amino or carboxy terminal positions of the reference amino acid sequence or anywhere between those terminal positions, interspersed either individually among residues in the reference sequence or in one or more contiguous groups within the reference sequence.

[0067] As a practical matter, whether any particular polypeptide is at least 80%, 85%, 90%, 92%, 95%, 96%, 97%, 98% or 99% identical to, for instance, the amino acid sequence shown in SEQ ID NO:2, 4, 6, 8, 10, 12, 14 or to the amino acid sequence encoded by a nucleic acid sequence can be determined conventionally using known computer programs such the Bestfit program (Wisconsin Sequence Analysis Package, Version 8 for Unix, Genetics Computer Group, University Research Park, 575 Science Drive, Madison, WI 53711). When using Bestfit or any other sequence alignment program to determine

whether a particular sequence is, for instance, 95% identical to a reference sequence according to the present invention, the parameters are set, of course, such that the percentage of identity is calculated over the full length of the reference amino acid sequence and that gaps in homology of up to 5% of the total number of amino acid residues in the reference sequence are allowed.

**[0068]** In a specific embodiment, the identity between a reference sequence (query sequence, a sequence of the present invention) and a subject sequence, also referred to as a global sequence alignment, is determined using the FASTDB computer program based on the algorithm of Brutlag *et al.* (Comp. App. Biosci. 6:237-245 (1990)). Preferred parameters used in a FASTDB amino acid alignment are: Matrix=PAM 0, k-tuple=2, Mismatch Penalty=1, Joining Penalty=20, Randomization Group Length=0, Cutoff Score=1, Window Size=sequence length, Gap Penalty=5, Gap Size Penalty=0.05, Window Size=500 or the length of the subject amino acid sequence, whichever is shorter. According to this embodiment, if the subject sequence is shorter than the query sequence due to N- or C-terminal deletions, not because of internal deletions, a manual correction is made to the results to take into consideration the fact that the FASTDB program does not account for N- and C-terminal truncations of the subject sequence when calculating global percent identity. For subject sequences truncated at the N- and C-termini, relative to the query sequence, the percent identity is corrected by calculating the number of residues of the query sequence that are N- and C-terminal of the subject sequence, which are not matched/aligned with a corresponding subject residue, as a percent of the total bases of the query sequence. A determination of whether a residue is matched/aligned is determined by results of the FASTDB sequence alignment. This percentage is then subtracted from the percent identity, calculated by the above FASTDB program using the specified parameters, to arrive at a final percent identity score. This final percent identity score is what is used for the purposes of this embodiment. Only residues to the N- and C-termini of the subject sequence, which are not matched/aligned with the query sequence, are considered for the purposes of manually adjusting the

percent identity score. That is, only query residue positions outside the farthest N- and C-terminal residues of the subject sequence. For example, a 90 amino acid residue subject sequence is aligned with a 100 residue query sequence to determine percent identity. The deletion occurs at the N-terminus of the subject sequence and therefore, the FASTDB alignment does not show a matching/alignment of the first 10 residues at the N-terminus. The 10 unpaired residues represent 10% of the sequence (number of residues at the N- and C-termini not matched/total number of residues in the query sequence) so 10% is subtracted from the percent identity score calculated by the FASTDB program. If the remaining 90 residues were perfectly matched the final percent identity would be 90%. In another example, a 90 residue subject sequence is compared with a 100 residue query sequence. This time the deletions are internal deletions so there are no residues at the N- or C-termini of the subject sequence which are not matched/aligned with the query. In this case the percent identity calculated by FASTDB is not manually corrected. Once again, only residue positions outside the N- and C-terminal ends of the subject sequence, as displayed in the FASTDB alignment, which are not matched/aligned with the query sequence are manually corrected for.

### C. Methods and Processes of the Invention

**[0069]** Various embodiments of the invention provide methods of utilizing the KDB, KDBH, KDB2 and KDB2HL polynucleotide molecules. In a preferred embodiment, any one of these polynucleotide molecules is utilized to increase the production of lysine from a host cell.

**[0070]** The amino acid pathway for L-lysine biosynthesis is well known to skilled artisans of amino acid production. Genes encoding the enzymes important for the conversion of L-aspartate to L-lysine include the *ask*, *asd*, *dapA*, *dapB*, *ddh* and *lysA* genes (Figure 1). Thus, the invention provides herein specific embodiments utilizing L-lysine biosynthetic pathway genes.

[0071] The isolated polynucleotide molecules of the invention are preferably propagated and maintained in an appropriate nucleic acid vector. Methods for the isolation and cloning of the isolated nucleic acid molecules of the invention are well known to those skilled in the art of recombinant DNA technology. Appropriate vectors and methods for use with prokaryotic and eukaryotic hosts are described by Sambrook *et al.*, *Molecular Cloning: A Laboratory Manual*, Second Edition, Cold Spring Harbor, N.Y., 1989, the disclosure of which is hereby incorporated by reference.

[0072] A great variety of vectors can be used in the invention. Such vectors include chromosomal, episomal and virus-derived vectors, *e.g.*, vectors derived from bacterial plasmids and from bacteriophage, as well as vectors derived from combinations thereof, such as those derived from plasmid and bacteriophage genetic elements, such as cosmids and phagemids, all can be used in accordance with this aspect of the present invention. Retroviral vectors can be replication competent or replication defective. In the latter case, viral propagation generally will occur only in complementing host cells. Preferred, are vectors suitable to maintain and propagate a polynucleotide in a bacterial host.

[0073] A large numbers of suitable vectors and promoters for use in bacteria are known, many of which are commercially available. Preferred prokaryotic vectors include plasmids such as those capable of replication in *E. coli* (such as, for example, pBR322, ColEI, pSC101, pACYC 184,  $\pi$ VX). Such plasmids are, for example, disclosed by Maniatis, T., *et al.*, *In: Molecular Cloning, A Laboratory Manual*, Cold Spring Harbor Press, Cold Spring Harbor, NY (1982)). The following vectors are provided by way of example: pET (Novagen), pQE70, pQE60, pQE-9 (Qiagen), pBs, phagescript, psiXI74, pBlueScript SK, pBsKS, pNH8a, pNH16a, pNH18a, pNH46a (Stratagene), pTrc99A, pKK223-3, pKK233-3, pDR540, pRIT5 (Pharmacia).

[0074] Preferred vectors for the isolated nucleic acid molecules of the invention include the pFC1 to pFC7 novel family of combinatorial cloning vectors (Lonsdale, D.M., *et al.*, *Plant Molecular Biology Reporter* 13: 343-345 (1995))

and the pK184 vector (Jobling, M.G. and Homes, R.K., *Nucleic Acid Research* 18: 5315-5316 (1990)).

[0075] Another group of preferred vectors are those that are capable of autonomous replication in *Corynebacterium* species. Such vectors are well known to those skilled in the art of amino acid production by way of microbial fermentation, examples of which include pSR1, pMF1014 $\alpha$  and vectors derived therefrom. Other suitable vectors will be readily apparent to the skilled artisan.

[0076] A KDB, KDBH, KDB2 or KDB2HL polynucleotide can be joined to a vector containing a selectable marker for propagation in a host. The vectors can include at least one selectable marker. In this regard, vectors preferably contain one or more selectable marker genes to provide a phenotypic trait for selection of transformed host cells. Such markers include dihydrofolate reductase, G418 or neomycin resistance for eukaryotic cell culture and tetracycline, kanamycin or ampicillin resistance genes, or an autotrophic gene which allows the host cell to grow in the absence of a nutrient for which the host cell strain is normally autotrophic.

[0077] Representative examples of appropriate hosts include, but are not limited to, bacterial cells, such as *E. coli*, *Streptomyces* and *Salmonella typhimurium* cells; fungal cells, such as yeast cells and insect cells such as *Drosophila* S2 and *Spodoptera* Sf9 cells. Appropriate culture mediums and conditions for the above-described host cells are known in the art.

[0078] If the vector is intended to be maintained in the host cell extrachromosomally, it will contain, in addition an origin of replication which will allow it to replicate in the host cell. Alternatively, if it is desired that the vector integrate into the chromosome, the vector is constructed such that it cannot replicate in the host cell. For example, such a vector might be capable of propagation in another organism, for example, *E. coli*, but lack the proper origin of replication to be propagated in *Corynebacterium*. In another aspect of this embodiment, the vector is a shuttle vector which can replicate and be maintained in more than one host cell species, for example, such a shuttle vector might be

capable of replication in a *Corynebacterium* host cell such as a *C. glutamicum* host cell, and also in an *E. coli* host cell.

[0079] In one embodiment of the invention, the additional copies of the L-lysine biosynthesis pathway gene(s) selected from *ask*, *asd*, *dapB*, *ddh*, *ORF2* and *lysA* can be integrated into the chromosome. Another embodiment of the invention provides that the additional copies of the L-lysine biosynthesis pathway gene(s) are carried extra-chromosomally. Amplifications by a factor of 5 or less can be obtained by introducing the additional gene copies into the chromosome of the host strain by way of single event homologous recombination. In a most preferred embodiment, the recombination event results in the introduction of one additional copy of the copy of the gene or genes of interest. If more than 5 copies of the genes are desired, multicopy plasmids carrying the recombinant DNA construct of the invention can be utilized.

[0080] In another embodiment of the invention, enzyme activity is increased by overexpressing one or more genes of the group comprising *ask*, *asd*, *dapB*, *ddh*, *ORF2* and *lysA* encoding one or more lysine biosynthetic pathway enzymes. In one embodiment of the invention, said one or more genes are operably linked directly or indirectly to one or more promoter sequences. In another embodiment of the invention, said operably linked promoter sequences are heterologous, endogenous, or hybrid. In a preferred embodiment of the invention, said promoter sequences are one or more of: a promoter sequence from the 5' end of genes endogenous to *C. glutamicum*, a promoter sequence from plasmids that replicate in *C. glutamicum*, and, a promoter sequence from the genome of phage which infect *C. glutamicum*. In another embodiment, one or more of said promoter sequences are modified. In another preferred embodiment, said modification comprises truncation at the 5' end, truncation at the 3' end, non-terminal insertion of one or more nucleotides, non-terminal deletion of one or more nucleotides, addition of one or more nucleotides at the 5' end, addition of one or more nucleotides at the 3' end, and, combinations thereof. In a preferred embodiment,

the P1 promoter, the first promoter of the *argS-lysA* operon is used as the promoter for the *lysA* gene.

[0081] Alternative gene promoter elements can be utilized in the constructs of the invention. For example, known bacterial promoters suitable for this use in the present invention include the *E. coli lacI* and *lacZ* promoters, the *T3* and *T7* promoters, the *gpt* promoter, the lambda *PR* and *PL* promoters, the *trp* promoter, or promoters endogenous to the bacterial cells of the present invention. Other promoters useful in the invention include regulated promoters, unregulated promoters and heterologous promoters. Many such promoters are known to one of skill in the art. See Sambrook, E.F. *et al.*, *Molecular Cloning: A Laboratory Manual*, 2d ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York (1989).

[0082] In addition, the vector can contain control regions that regulate as well as engender expression. Generally, such regions will operate by controlling transcription, such as inducer or repressor binding sites and enhancers, among others.

[0083] In a preferred embodiment of the invention, the KDB polynucleotide molecule is encompassed in vector pDElia2<sub>FC5</sub>-KDB. In another preferred embodiment, the KDBH polynucleotide molecule is encompassed in vector pK184-KDBH. In another preferred embodiment, the KDB2 polynucleotide molecule is encompassed in vector pDElia2<sub>FC5</sub>-KDB2. In a further preferred embodiment, the KDB2HL polynucleotide is encompassed in vector pDElia2<sub>FC5</sub>-KDB2HL.

[0084] It is a further object of the invention to provide a host cell comprising a vector comprising any one of the isolated KDB, KDBH, KDB2 or KDB2HL polynucleotide molecule.

[0085] Introduction of the construct into the host cell can be effected by calcium phosphate transfection, DEAE-dextran mediated transfection, cationic lipid-mediated transfection, electroporation, transduction, infection, or other methods. Such methods are described in many standard laboratory manuals, such as Davis

et al., Basic Methods In Molecular Biology (1986). If the vector is a virus, it can be packaged in vitro using an appropriate packaging cell line and then transduced into host cells.

[0086] Representative examples of appropriate hosts for the above described isolated nucleic acid molecules include, but are not limited to, bacterial cells, such as *C. glutamicum*, *Escherichia coli*, *Streptomyces* and *Salmonella typhimurium* cells; and fungal cells, such as yeast cells. Appropriate culture media and conditions for the above-described host cells are known in the art.

[0087] Bacterial cells, such as *E. coli* and coryneform bacteria are preferred as host cells. Particularly preferred *Corynebacterium* and *Brevibacterium* species of the methods and processes of the invention include: *Corynebacterium glutamicum*, *Brevibacterium flavum*, *Brevibacterium lactofermentum* and other *Corynebacteria* and *Brevibacteria* species known in the art.

[0088] As will be understood by those skilled in the art, the term “*Corynebacterium* species” includes those organisms previously identified in the literature as “*Brevibacterium* species,” for example *Brevibacterium flavum* and *Brevibacterium lactofermentum* which have now been reclassified into the genus *Corynebacterium* (*Int. J. Syst. Bacteriol.* 41: 255 (1981)).

[0089] It is a further object to provide a host cell wherein said host cell is a *Brevibacterium* selected from the group consisting of *Brevibacterium flavum* NRRL-B30218, *Brevibacterium flavum* NRRL-B30458, *Brevibacterium flavum* NRRL-B30410, *Brevibacterium flavum* NRRL-B30459, *Brevibacterium flavum* NRRL-B30522, *Brevibacterium flavum* NRRL-B30219, *Brevibacterium lactofermentum* NRRL-B30220, *Brevibacterium lactofermentum* NRRL-B30221, *Brevibacterium lactofermentum* NRRL-B30222, *Brevibacterium flavum* NRRL-30234 and *Brevibacterium lactofermentum* NRRL-30235. In another embodiment, the host cell is *Escherichia coli*. In a preferred embodiment, the host cell is *E. coli* DH5  $\alpha$  MCR NRRL-B30228. In another embodiment, the host cell is a *C. glutamicum* selected from the group consisting of *C. glutamicum* NRRL-B30236 and *C. glutamicum* NRRL-B30237.



[0090] The methods to increase the production of lysine and the processes for the production of lysine of the invention can both utilize a step requiring the transformation of an isolated nucleic acid molecule into a host cell.

[0091] The methods to increase the production of lysine and the processes for the production of lysine of the invention can utilize a step requiring amplification of at least one lysine biosynthesis pathway gene. As known to one skilled in the art, the term amplification means increasing the number of a gene or genes of lysine biosynthetic pathway by any means known in the art. Particularly preferred means of amplification include: (1) the addition an isolated KDB, KDBH, KDB2 or KDB2HL polynucleic acid molecule by insertion into the chromosome of a host cell, for example by homologous recombination, and (2) the addition an isolated KDB, KDBH, KDB2 or KDB2HL polynucleic acid molecule into a host cell by way of a self-replicating, extra-chromosomal vector, for example, a plasmid.

[0092] Methods of inserting an isolated nucleic acid molecule into the chromosome of a host cell are known to those skilled in the art. For example, insertion of isolated nucleic acid molecules into the chromosome of *Corynebacterium* species can be done utilizing the pK184 plasmid described by Jobling, M. *et al.*, *Nucleic Acids Research* 18(17): 5315-5316 (submitted 1990). Because these vectors lack a *Corynebacterium* species origin of replication and contain a selectable marker such as kanamycin (*kan*), cells will only be capable of growing under selection if the vector has been inserted into the host cell chromosome by homologous recombination.

[0093] In alternative embodiments, the invention also provides methods for increasing lysine production and processes for the production of lysine wherein biosynthetic pathway gene amplification is accomplished through the introduction into a host cell of a self-replicating, extra-chromosomal vector, *e.g.*, a plasmid, comprising an isolated KDB, KDBH, KDB2 or KDB2HL polynucleotide molecule. Suitable plasmids for these embodiments include pSR1 and other derivatives of pSR1 (Archer, J. *et al.*, *J. Gen. Microbiol.* 139: 1753-1759 (1993)).

[0094] For various embodiments of the invention drawn to a method to increase production of L-lysine, screening for increased production of L-lysine, can be determined by directly comparing the amount of L-lysine produced in culture by a *Corynebacterium* species host strain to that of a *Corynebacterium* species transformed host strain in which lysine biosynthesis gene or genes are amplified. The level of production of lysine can conveniently be determined by the following formula to calculate the percent yield from dextrose:  $[(\text{g lysine/L} / (\text{g dextrose consumed/L})) \times 100]$ .

[0095] In one embodiment, the invention provides a method to increase the production of lysine comprising: (a) transforming a host cell with an isolated KDBH polynucleotide molecule (b) selecting a transformed host cell; and (c) screening for increased production of lysine from said transformed host cell relative to said host cell. In another embodiment of the method, the method further comprises growing said transformed host cell in a medium; and purifying lysine produced by said transformed host cell.

[0096] A variety of media known to those skilled in the art can be used to support cell growth for the production of lysine. Illustrative examples of suitable carbon sources include, but are not limited to: carbohydrates, such as glucose, fructose, sucrose, starch hydrolysate, cellulose hydrolysate and molasses; organic acids, such as acetic acid, propionic acid, formic acid, malic acid, citric acid, and fumaric acid; and alcohols, such as glycerol. Illustrative examples of suitable nitrogen sources include, but are not limited to: ammonia, including ammonia gas and aqueous ammonia; ammonium salts of inorganic or organic acids, such as ammonium chloride, ammonium phosphate, ammonium sulfate and ammonium acetate; and other nitrogen-containing sources, including meat extract, peptone, corn steep liquor, casein hydrolysate, soybean cake hydrolysate, urea and yeast extract.

[0097] A variety of fermentation techniques are known in the art which can be employed in processes of the invention drawn to the production of amino acids. Generally, amino acids can be commercially produced from the invention in

fermentation processes such as the batch type or of the fed-batch type. In batch type fermentations, all nutrients are added at the beginning of the fermentation. In fed-batch or extended fed-batch type fermentations one or a number of nutrients are continuously supplied to the culture, right from the beginning of the fermentation or after the culture has reached a certain age, or when the nutrient(s) which are fed were exhausted from the culture fluid. A variant of the extended batch of fed-batch type fermentation is the repeated fed-batch or fill-and-draw fermentation, where part of the contents of the fermenter is removed at some time, for instance when the fermenter is full, while feeding of a nutrient is continued. In this way a fermentation can be extended for a longer time.

[0098] Another type of fermentation, the continuous fermentation or chemostat culture, uses continuous feeding of a complete medium, while culture fluid is continuously or semi-continuously withdrawn in such a way that the volume of the broth in the fermenter remains approximately constant. A continuous fermentation can in principle be maintained for an infinite time.

[0099] In a batch fermentation an organism grows until one of the essential nutrients in the medium becomes exhausted, or until fermentation conditions become unfavorable (*e.g.*, the pH decreases to a value inhibitory for microbial growth). In fed-batch fermentations measures are normally taken to maintain favorable growth conditions, *e.g.*, by using pH control, and exhaustion of one or more essential nutrients is prevented by feeding these nutrient(s) to the culture. The microorganism will continue to grow, at a growth rate dictated by the rate of nutrient feed. Generally a single nutrient, very often the carbon source, will become limiting for growth. The same principle applies for a continuous fermentation, usually one nutrient in the medium feed is limiting, all other nutrients are in excess. The limiting nutrient will be present in the culture fluid at a very low concentration, often unmeasurably low. Different types of nutrient limitation can be employed. Carbon source limitation is most often used. Other examples are limitation by the nitrogen source, limitation by oxygen, limitation by a specific nutrient such as a vitamin or an amino acid (in case the microorganism is

auxotrophic for such a compound), limitation by sulphur and limitation by phosphorous.

**[0100]** Lysine can be recovered by any method known in the art. Exemplary procedures are provided in the following: Van Walsem, H.J. & Thompson, M.C., *J. Biotechnol.* 59:127-132 (1997), and U.S. Pat. No. 3,565,951, both of which are incorporated herein by reference.

**[0101]** The pDElia2<sub>FC5</sub>-KDB, the pK184-KDBH, the pDElia2<sub>FC5</sub>-KDB2 and the pDElia2<sub>FC5</sub>-KDB2HL constructs in NRRL-B11474 host cells were deposited at an acceptable International Depositary Authority in accordance with the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purposes of Patent Procedure. The deposits have been made with the Agricultural Research Service, Culture Collection (NRRL), 1815 North University Street, Peoria, Illinois 61604.

**[0102]** All patents and publications referred to herein are expressly incorporated by reference in their entirety.

## EXAMPLES

### EXAMPLE 1

#### Preparation of L-Lysine Pathway Multi-gene Constructs

**[0103]** Constructs comprising a KDB, a KDBH, a KDB2HL or a KDB2 polynucleotide molecule were made from the following sources:

<i>Gene(s)</i>	<i>Source</i>
<i>ask-asd</i>	Strain ATCC 21529;
<i>dapB</i>	Strain NRRL B11474;
<i>ddh</i>	Strain NRRL B11474;
<i>ORF2</i>	Strain NRRL B11474;
<i>lysA</i>	Strain ASO19;
<b>Promoter</b>	
<i>P1</i>	<i>argS-lysA</i> operon from pRS6

[0104] The polymerase chain reaction (PCR) technique was used to construct the KDB, KDBH, KDB2, and KDBHL constructs. Standard PCR and subcloning procedures were utilized in cloning the coding regions of *ask-asd*, *dapB-ORF2-dapA*, *ddh*.

[0105] The primers utilized for cloning experiments included:

<i>Primer name</i>	<i>Sequence</i>
<i>ask</i>	5'-GGGTACCTCGCGAAGTAGCACCTGTCAC-3'
<i>asd</i>	5'-GCGGATCCCCCATCGCCCCTCAAAGA-3'
<i>dapB</i>	5'-AACGGGCGGTGAAGGGCAACT-3'
<i>ORF2</i>	5'-GCTCATAGAGTTCAAGGTTACCTTCTTCCC-3'
<i>ddh1</i>	5'-CCATGGTACCAAGTGCGTGGCGAG-3'
<i>ddh2</i>	5'-CCATGGTACCACACTGTTTCCTTGC-3'
<i>lysA<sub>(ATG)</sub></i>	5'-CCGGAGAAGATGTAACAATGGCTAC-3'
<i>lysA3B</i>	5'-CCTCGACTGCAGACCCCTAGACACC-3'
<i>dapA</i>	5'-TGAAAGACAGGGGTATCCAGA-3'

[0106] Construction procedures and intermediate plasmids are described in Figures 11-14. The following steps (Figure 11) were performed in constructing the pDELia2<sub>FC5</sub>-KDB vector:

1. pGEMT-ask-asd: an approximately 2.6 Kb PCR product containing the *ask-asd* operon of ATCC21529 using primers *ask* and *asd* was cloned into pGEM-T (Promega pGEM-T vector systems).
2. pFC3-ask-asd: an approximately 2.6 Kb NsiI-ApaI fragment of pGEMT-ask-asd was cloned into pFC3 cut with PstI and ApaI.
3. pFC3-dapB-ORF2-dapA: an approximately 2.9 Kb PCR product of NRRL-B11474 *dapB-ORF2-dapA* coding region was cloned into pFC3 at the EcoRV site.
4. pFC3-dapB: the large ClaI fragment of pFC3-dapB-ORF2-dapA was religated.
5. pUC18-ddh: an approximately 1.3 Kb KpnI fragment of pADM21 containing *ddh* (NRRL-B11474 locus) was subcloned into pUC18 at the KpnI site.
6. pFC1-ddh: an approximately 1.3 Kb SalI-EcoRI fragment of pUC18-ddh was cloned into pFC1 cut with SalI and EcoRI.
7. pFC1-ddh-lysA: an approximately 2.1 Kb EcoRI-PstI fragment (containing the intact *lysA* DNA) of pRS6 was cloned into pFC1-ddh cut with EcoRI and PstI.
8. pFC1-ask-asd-ddh-lysA: an approximately 2.6 Kb SwaI-FseI fragment of pFC3-ask-asd was cloned into pFC1-ddh-lysA cut with SwaI and FseI.
9. pFC3-ask-asd-dapB-ddh-lysA: an approximately 6 Kb SpeI fragment of pFC1-ask-asd-ddh-lysA was cloned into pFC3-dapB at the SpeI site.
10. pDELia2<sub>FC5</sub>-ask-asd-dapB-ddh-lysA (pDELia2<sub>FC5</sub>-KDBHL): an approximately 7.38 Kb NotI-PmeI fragment of pFC3-ask-asd-dapB-ddh-lysA was cloned into pDELia2<sub>FC5</sub> cut with NotI and PmeI.

11. pDElia2: an approximately 1.24 Kb blunted PstI fragment of pUC4K was ligated with the approximately 1.75 Kb DraI-SspI fragment of pUC19.

12. pDElia2<sub>FC5</sub>: the small PvuII fragment of pFC5 was ligated with the large PvuII fragment of pDElia2.

13. pDElia2<sub>FC5</sub>-ask-asd-dapB (pDElia2<sub>FC5</sub>-KDB): an approximately 4 Kb ApaI fragment of pDElia2<sub>FC5</sub>-KDBHL was cloned into pDElia2<sub>FC5</sub> at the ApaI site.

[0107] Corynebacterium (NRRL-B11474) containing the pDElia2<sub>FC5</sub>-KDB construct was deposited at an acceptable International Depository Authority in accordance with the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purposes of Patent Procedure. The deposit has been made with the Agricultural Research Service, Culture Collection (NRRL), 1815 North University Street, Peoria, Illinois 61604 on February 1, 2001. The deposit is numbered NRRL-B30458.

[0108] The following steps (Figure 12) were preformed in constructing the pK184-KDBH construct:

1. pGEMT-ask-asd: an approximately 2.6 Kb PCR product containing the *ask-asd* operon of ATCC21529 using primers *ask* and *asd* was cloned into pGEM-T (Promega pGEM-T vector systems).

2. pFC3-ask-asd: an approximately 2.6 Kb NsiI-ApaI fragment of pGEMT-ask-asd was cloned into pFC3 cut with PstI and ApaI.

3. pFC3-ask-asd-ddh: an approximately 1.3 Kb KpnI fragment containing NRRL-B11474 *ddh* was cloned into pFC3-ask-asd at the KpnI site.

4. pFC3-dapB-ORF2-dapA: an approximately 2.9 Kb PCR product of NRRL-B11474 *dapB-ORF2-dapA* coding region was cloned into pFC3 at the EcoRV site.

5. pFC3-dapB: the large ClaI fragment of pFC3-dapB-ORF2-dapA was religated.

6. pFC3-ask-asd-dapB-ddh: an approximately 4 Kb NotI-SmaI fragment of pFC3-ask-asd-ddh was cloned into pFC3-dapB digested with NotI and SmaI.

7. pK184-ask-asd-dapB-ddh (pK184-KDBH): an approximately 5.3 Kb PmeI fragment containing ask-asd-dapB-ddh was cloned into pK184 at the SmaI site.

[0109] Corynebacterium (NRRL-B11474) containing the pK184-KDBH construct was deposited at an acceptable International Depositary Authority in accordance with the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purposes of Patent Procedure. The deposit has been made with the Agricultural Research Service, Culture Collection (NRRL), 1815 North University Street, Peoria, Illinois 61604 on February 1, 2001. The deposit is numbered NRRL-B30410.

[0110] The following steps (Figure 13) were performed in constructing the pDElia2<sub>FC5</sub>-KDB2 vector:

1. pGEMT-ask-asd: an approximately 2.6 Kb PCR product containing the ask-asd operon of ATCC21529 using primers ask and asd was cloned into pGEM-T (Promega pGEM-T vector systems).

2. pUC18-ddh: an approximately 1.3 Kb KpnI fragment of pADM21 containing ddh (BF100 locus) was subcloned into pUC18 at the KpnI site.

3. pFC3-ask-asd: an approximately 2.6 Kb NsiI-ApaI fragment of pGEMT-ask-asd was cloned into pFC3 cut with PstI and ApaI

4. pFC1-dapB-ORF2: an approximately 2 Kb PCR product of NRRL-B11474 dapB-ORF2 coding region was cloned into pFC1 at the EcoRV site.

5. pFC1-ddh: an approximately 1.3 Kb PstI-EcoRI fragment of pUC18-ddh was cloned into pFC1 cut with PstI and EcoRI.



6. pUC19-P1: an approximately 550 bp HpaI-PvuII fragment (containing the first promoter, P1, of the argS-lysA operon) of pRS6 was cloned into pUC19 at the SmaI site.

7. pUC19-P1lysA: an approximately 1.45 Kb promoterless PCR product, using primers LysA(ATG) and LysA3B, of ASO19 lysA coding region is cloned into pUC19-P1 at the HincII site.

8. pFC1-P1lysA: an approximately 2 Kb EcoRI-HindIII fragment of pUC19-P1lysA was cloned in to pFC1 cut with EcoRI and HindIII.

9. pFC1-ddh-P1lysA: an approximately 1.3 Kb EcoRI-NotI fragment of pFC1-ddh was cloned into pFC1-P1lysA cut with EcoRI and NotI.

10. pFC1-ask-asd-ddh-P1lysA: an approximately 2.6 Kb SmaI-FseI fragment of pFC3-asd-asd was cloned into pFC1-ddh-P1lysA cut with SmaI and FseI.

10. pFC1-ask-asd-dapB-ORF2-ddh-P1lysA (pFC1-KDB2HPIL): an approximately 5.9 Kb SpeI fragment of pFC1-ask-asd-ddh-P1lysA was cloned into pFC1-dapB-ORF2 at the SpeI site.

11. pDElia2<sub>FC5</sub>: the small PvuII fragment of pFC5 was ligated with the large PvuII fragment of pDElia2.

12. pDElia2<sub>FC5</sub>-ask-asd-dapB-ORF2 (pDElia2<sub>FC5</sub>-KDB2): an approximately 4.7 Kb ApaI fragment containing KDB2 of pFC1-KDB2HPIL was cloned into pDElia2<sub>FC5</sub> at the ApaI site.

**[0111]** Corynebacterium (NRRL-B11474) containing the pDElia2<sub>FC5</sub>-KDB2 construct was deposited at an acceptable International Depositary Authority in accordance with the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purposes of Patent Procedure. The deposit has been made with the Agricultural Research Service, Culture Collection (NRRL), 1815 North University Street, Peoria, Illinois 61604 on February 1, 2001. The deposit is numbered NRRL-B30459.

[0112] The following steps (Figure 14) were performed in constructing the pDElia2<sub>FC5</sub>-KDB2HP1L vector:

1. pGEMT-ask-asd: an approximately 2.6 Kb PCR product containing the ask-asd operon of ATCC21529 using primers ask and asd was cloned into pGEM-T (Promega pGEM-T vector systems).
2. pUC18-ddh: an approximately 1.3 Kb KpnI fragment of pADM21 containing ddh (NRRL-B11474 locus) was subcloned into pUC18 at the KpnI site.
3. pFC3-ask-asd: an approximately 2.6 Kb NsiI-ApaI fragment of pGEMT-ask-asd was cloned into pFC3 cut with PstI and ApaI.
4. pFC1-dapB-ORF2: an approximately 2 Kb PCR product of NRRL-B11474 dapB-ORF2 coding region was cloned into pFC1 at the EcoRV site.
5. pFC1-ddh: an approximately 1.3 Kb PstI-EcoRI fragment of pUC19-ddh was cloned into pFC1 cut with PstI and EcoRI.
6. pUC19-P1: an approximately 550 bp HpaI-PvuII fragment (containing the first promoter, P1, of the *argS-lysA* operon) of pRS6 was cloned into pUC19 at the SmaI site.
7. pUC19-P1lysA: an approximately 1.45 Kb promoterless PCR product, using primers LysA(ATG) and LysA3B, of ASO19 lysA coding region is cloned into pUC19-P1 at the HincII site.
8. pFC1-P1lysA: an approximately 2 Kb EcoRI-HindIII fragment of pUC19-P1lysA was cloned into pFC1 cut with EcoRI and HindIII.
9. pFC1-ddh-P1lysA: an approximately 1.3 Kb EcoRI-NotI fragment of pFC1-ddh was cloned into pFC1-P1lysA cut with EcoRI and NotI.
10. pFC1-ask-asd-ddh-P1lysA: an approximately 2.6 Kb SwaI-FseI fragment of pFC3-ask-asd was cloned into pFC1-ddh-P1lysA cut with SwaI and FseI.

11. pFC1-ask-asd-dapB-ORF2-ddh-P1lysA (pFC1-KDB2HPIL): an approximately 5.9 Kb SpeI fragment of pFC1-ask-asd-ddh-P1lysA was cloned into pFC1-dapB-ORF2 at the SpeI site.

12. pDElia2<sub>FC5</sub>: the small PvuII fragment of pFC5 was ligated with the large PvuII fragment of pDElia2

13. pDElia2<sub>FC5</sub>-ask-asd-dapB-ORF2-ddh-P1lysA (pDElia2<sub>FC5</sub>-KDB2HP1L): an approximately 7.9 Kb NHE fragment of pFC1-ask-asd-dapB-ORF2-ddh-P1lysA was cloned into pDElia2<sub>FC5</sub> at the NHE site.

[0113] Corynebacterium (NRRL-B11474) containing the pDElia2<sub>FC5</sub>-KDB2HP1L construct was deposited at an acceptable International Depositary Authority in accordance with the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purposes of Patent Procedure. The deposit has been made with the Agricultural Research Service, Culture Collection (NRRL), 1815 North University Street, Peoria, Illinois 61604 on February 1, 2001. The deposit is numbered NRRL-B30522.

## EXAMPLE 2

### Screening and Selection of Strains with Improved L-Lysine Production

[0114] The production of L-lysine by cells stably transformed with multi-gene constructs is summarized in Table 1.

**Table 1. Lysine production by various parental and stably transfected bacteria**

Strain Tested	lysine titer (g/L)	L-lysine yield (%)	Cell Deposit
NRRL-B11474	31	30	
NRRL-B11474::pDElia2 <sub>FC5</sub> -KDB	34	37	NRRL-B30458

Strain Tested	lysine titer (g/L)	L-lysine yield (%)	Cell Deposit
NRRL-B11474	31	31	
NRRL-B11474::pK184-KDBH	38	37.4	NRRL-B30410
NRRL-B11474	30	30	
NRRL-B11474::pDElia2 <sub>FC5</sub> -KDB2	39	37	NRRL-B30459
NRRL-B11474	31	33	
NRRL-B11474::pDElia2 <sub>FC5</sub> -KDB2HP1L	38	41	NRRL-B30522

\* \* \* \* \*

[0115] Having now fully described the present invention in some detail by way of illustration and example for purposes of clarity of understanding, it will be obvious to one of ordinary skill in the art that same can be performed by modifying or changing the invention with a wide and equivalent range of conditions, formulations and other parameters thereof, and that such modifications or changes are intended to be encompassed within the scope of the appended claims.

[0116] All publications, patents and patent applications mentioned in this specification are indicative of the level of skill of those skilled in the art to which this invention pertains, and are herein incorporated by reference to the same extent as if each individual publication, patent or patent application was specifically and individually indicated to be incorporated by reference.